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METHOD FOR STUDYING PROTEIN INTERACTIONS *IN VIVO*

BACKGROUND

The study of interactions between proteins in living cells is often necessary to understand the proteins' functions and their mechanisms of action. These interactions are currently studied using immuno-precipitation, the yeast two hybrid method, and β-gal complementation method.

However, these methods are associated with several disadvantages. For example, these methods are associated with false positives. Second, they do not permit the determination of quantitative information regarding the interactions. Further, they do not allow for *in vivo* real time monitoring of the interactions.

Therefore, it would be advantageous to have another method of studying interactions between proteins *in vivo*, which does not have these disadvantages. Further preferably, the method could be used with a wide variety of proteins and in a wide variety of living cells. Also preferably, the method could be used to determine the interactions between molecules other than proteins.

SUMMARY

According to one embodiment of the present invention, there is provided a

method for determining whether a first protein interacts with a second protein within a living cell. The method comprises providing the first protein complexed to a donor luciferase and the second protein complexed to an acceptor fluorophore within the cell. The donor luciferase is capable of luminescence resonance energy transfer to the acceptor fluorophore when the first protein is in proximity to the second protein. Then, the complexed first protein and the complexed second protein are allowed to come into proximity to each other within the cell. Next, any fluorescence from the acceptor fluorophore is detected. Fluorescence of the acceptor fluorophore resulting from luminescence resonance energy transfer from the donor luciferase to acceptor fluorophore the indicates that the first protein has interacted with the second protein.

In a preferred embodiment, providing the first protein complexed to a donor luciferase and the second protein complexed to an acceptor fluorophore comprises genetically engineering DNA and transferring the genetically engineered DNA to the living cell causing the cell to produce the first protein complexed to a donor luciferase and the second protein complexed to an acceptor fluorophore. In a particularly preferred embodiment, the cell which is provided with the first protein complexed to a donor luciferase and the cell which is provided with the second protein complexed to an acceptor fluorophore are mammalian cells.

In another preferred embodiment, the donor luciferase provided is *Renilla* luciferase. In yet another preferred embodiment, the acceptor fluorophore provided is an *Aequorea* green fluorescent protein.

In a particularly preferred embodiment, the detection of acceptor fluorophore fluorescence is performed using spectrofluorometery.

DESCRIPTION

The present invention includes a method for determining whether a first protein interacts with a second protein in a living cell using luminescent resonance energy transfer (LRET). Luminescence resonance energy transfer results from the transfer of excited state energy from a donor luciferase to an acceptor fluorophore. In order for LRET to occur, there must be an overlap between the emission spectrum of the donor luciferase and the excitation spectrum of the acceptor fluorophore.

The efficiency of luminescence resonance energy transfer is dependent on the distance separating the donor luciferase and the acceptor fluorophore, among other variables.

Generally, significant energy transfers occur only where the donor luciferase and acceptor fluorophore are less than about 80 Å of each other. This short distance is considerably less than the distance needed between for optical resolution between two entities using conventional microscopy. Therefore, detecting luminescence resonance energy transfer between a donor luciferase and an acceptor fluorophore indicates that the donor luciferase and acceptor fluorophore have come within the distance needed for LRET to occur, that is less than about 80 Å of each other.

The present invention utilizes luminescence resonance energy transfer to determine whether an interaction takes place between a first protein and a second protein in a living cell. This is accomplished by complexing a first protein to the donor luciferase and complexing the second protein to the acceptor fluorophore and placing the complexed first protein and the complexed second protein in the cell under conditions suitable for an interaction between the first protein and the second protein to take place. If the first protein interacts with the second protein, the donor luciferase will come close enough to the acceptor fluorophore for luminescence resonance energy transfer to take place and the acceptor fluorophore will fluoresce. Detection of fluorescence from the acceptor fluorophore will, thereby, indicate that the first protein has interacted with the second protein. Advantageously, this method allows for the detection of interaction between the first protein and the second protein even though the interaction cannot be detected by optical methods such as conventional microscopy.

There are several advantages of using luminescent resonance energy transfer to detect the interaction between two proteins according to the present invention. First, the specific labeling of the proteins in living cells can be achieved through genetic engineering methods where the introduction of fluorescent dyes into living cells is very difficult. Further, fluorescent dyes photobleach quickly while light emission of a luciferase such as *Renilla* luciferase originates from an enzymatic reaction that is relatively stable if substrate and oxygen are supplemented.

As used in this disclosure, "complexing a first protein to the donor luciferase" refers to joining the donor luciferase to the first protein in a manner that the donor luciferase and the first protein stay in essentially the same proximity to one another during interaction between the first protein and the second protein. Similarly, "complexing a second protein to

the acceptor fluorophore" refers to joining the acceptor fluorophore to the second protein in a manner that the acceptor fluorophore and the second protein stay in essentially the same proximity to one another during interaction between the first protein and the second protein. Such complexing can be done, for example, by genetically engineering the cell to produce a fusion protein containing the donor luciferase and first protein, and the acceptor fluorophore and the second protein.

In a preferred embodiment, the present invention uses *Renilla* luciferase as the donor luciferase and "humanized" *Aequorea* green fluorescent protein ('humanized' GFP) as the acceptor fluorophore. *Renilla* luciferase is a 34 kDa enzyme purified from *Renilla reniformis*. The enzyme catalyzes the oxidative decarboxylation of coelenterazine in the presence of oxygen to produce blue light with an emission wavelength maximum of 471 nm. *Renilla* luciferase was used as the donor luciferase because it requires an exogenous substrate rather than exogenous light for excitation. This, advantageously, eliminates background noise from an exogenous light source and from autofluorescence, and allows easy and accurate quantitative determination of light production.

'Humanized' GFP is a 27 kDa protein fluorophore that has an excitation maximum at 480 nm. It has a single amino acid difference from wild-type *Aequorea* green fluorescent protein. 'Humanized' GFP was chosen as the acceptor fluorophore because its excitation spectrum overlaps with the emission spectra of *Renilla* luciferase. Additionally, emissions from 'humanized' GFP can be visualized in living cells. Further, 'humanized' GFP is expressed well in the mammalian cells transfected with 'humanized' GFP cDNA that were used to demonstrate this method.

The method for determining whether a first protein interacts with a second protein according to the present invention was demonstrated as follows. In summary, insulin-like growth factor binding protein 6 (IGFBP 6) and insulin-like growth factor II (IGF-II) were selected as the first protein and second protein. IGFBP 6 is a protein known to have a marked binding affinity for IGF-II.

The *Renilla* luciferase cDNA was fused to IGFBP 6 cDNA and 'humanized' GFP cDNA was fused to IGF-II cDNA. Living cells were transfected with the fused cDNAs and the fusion proteins were expressed. Cell extracts were produced and mixed. The substrate for the Renilla luciferase moiety of the fused *Renilla* luciferase-IGFPB 6 protein

was added. Finally, fluorescence from the 'humanized' GFP moiety of the fused 'humanized' GFP-IGF-II protein was detected. Demonstration one method according to the present invention will now be described in greater detail.

A) The Cloning of Fused IGFBP-6 cDNA to *Renilla* Luciferase cDNA; Fused IGF-II cDNA to 'humanized' GFP cDNA; and Fused Insulin cDNA to 'humanized' GFP cDNA:

First, three fused cDNAs were produced: 1) fused IGFBP-6 cDNA and *Renilla* luciferase cDNA; 2) fused IGF-II cDNA and 'humanized' GFP cDNA; and 3) fused insulin cDNA and 'humanized' GFP cDNA. IGFBP-6 cDNA, SEQ ID NO:1, GenBank accession number M69054, encoded IGFBP-6, SEQ ID NO:2, which was used as the first protein. *Renilla* luciferase cDNA, SEQ ID NO:3, GenBank accession number M63501, encoded *Renilla* luciferase, SEQ ID NO:4, which was used as the donor luciferase. IGF-II cDNA, SEQ ID NO:5, encoded IGF-II, SEQ ID NO:6, which was used as the second protein. 'Humanized' GFP cDNA, SEQ ID NO:7, GenBank accession numberU50963, encoded 'humanized' GFP, SEQ ID NO:8,which was used as the acceptor fluorophore. Insulin cDNA, SEQ ID NO:9, accession number AH002844, encoded insulin, SEQ ID NO:10. Insulin, fused to 'humanized' GFP, was used as a control protein because insulin is homologous to IGF-II, but it does not bind to IGFBP-6. The IGFBP-6 cDNA, SEQ ID NO:1, IGF-II cDNA, SEQ ID NO:5, and insulin cDNA, SEQ ID NO:9, were modified using PCR as follows.

First, the cDNA of prepro-IGF-II carried on an EcoRI fragment was cloned into pBluescript KS (+) II vector. The insert was sequenced using T7 and T3 primers and confirmed to contain the known cDNA sequence of prepro-IGF-II. The 5' end of the IGF-II precursor was connected to the T7 promoter in the pBluescript KS (+) II vector. An IGF-II 3' primer was designed to generate a Notice of Allowance restriction site, to remove the D and E domains of prepro-IGF-II, and to maintain the Notice of Allowance fragment of the 'humanized' GFP in frame with the open reading frame of IGF-II.

Next, the IGF-II fragment was amplified with PCR using the T7 promoter primer and the IGF-II 3' primer. The PCR-amplified IGF-II fragment was digested by EcoRI and Not I and cloned into pCDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, US) producing pCDNA-IGF-II. Then, the Notice of Allowance fragment of the 'humanized' GFP was inserted into the Not I site of pCDNA-IGF-II producing pC-IGF-II-GFP.

The cDNA for precursor of insulin, which contained a signal peptide the B, C and A domains, was modified in a manner corresponding to the IGF-II fragment, above. The 'humanized' GFP cDNA was then linked to the 3' end of the modified insulin cDNA to produce pC-INS-GFP.

Finally, IGFBP 6 cDNA was amplified by PCR from a plasmid named Rat-tagged human IGFBP6. The stop codon of IGFBP 6 was removed and the open reading frame of IGFBP 6 was in frame with *Renilla* luciferase cDNA from pCEP4-RUC (Mayerhofer R, Langridge WHR, Cormier MG and Szalay AA. *Expression of recombinant Renilla luciferase in transgenic plants results in high levels of light emission.* The Plant Journal 1995;7;1031-8). The linking of the *Renilla* luciferase cDNA to the 3' end of modified IGFBP 6 cDNA produced pC-IGFBP 6-RUC.

The sequences of the insert DNA fragments from all the constructs were verified by DNA sequencing analysis. Qiagen Maxi Plasmid Kit (Qiagen, Inc., Valencia, CA) was used for the purification of plasmid DNA.

B) Transient Transfection of Mammalian Cells With pC-IGF-II-GFP, pC-INS-GFP and pC-IGFBP 6-RUC Using the Calcium Phosphate Precipitation Method:

Next, mammalian cells were transfected with the cloned fusion DNAs. First, COS-7 cells (African green monkey kidney cell, American Type Culture Collection CRL 1651) were grown at 37 C in Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine supplemented with 10% fetal bovine serum and antibiotic antimycotic solution containing a final concentration of penicillin 100 unit/ml, streptomycin 100 mg/ml and amphotericin B 250 ng/ml (Sigma-Aldrich Co., St. Louis, MO, US) in 5% CO_2. Groups of $1x10^6$ of these cells were plated the day before transfection and were approximately 50% to 60% confluent at the time of transfection.

Forty mg of each plasmid fusion DNA were precipitated and resuspended into Dulbecco's Phosphate Buffered Saline Solution and the plasmid fusion DNAs was introduced into mammalian cells using the standard calcium phosphate precipitation method. Transfection efficiency was estimated by fluorescence microscopy after 24 hours. The number of green fluorescent cells per plate were comparable in plates of pC-IGF-II-GFP DNA transfected cells, pC-INS-GFP DNA transfected cells and cells transfected with a plasmid DNA containing GFP only, which was used as a positive control.

C) Confirmation of Expression of Fusion Proteins:

Twenty-four hours after DNA transfection using DNA calcium phosphate precipitation method, individual plasmid DNA transfected COS-7 cells were visualized using fluorescence microscopy by detection of GFP fluorescence. pC-IGF-II-GFP and pC-INS-GFP transfected cells showed similar fluorescence patterns typical of secretory protein translocated through ER to Golgi. The pC-IGFBP 6-RUC transfected cells did not fluoresce. However, the pC-IGFBP 6-RUC transfected cells did show luminescence using a low light imaging system after the addition of coelenterazine.

Further, the presence of fusion proteins IGF-II-GFP and IGFBP 6-RUC, having the expected molecular weights of about 36 kDa and 56 kDa, respectively, were detected using immunoblot analysis. This confirmed the presence of both fusion proteins in the transiently transfected cells.

D) Detection of Protein Interactions by Spectrofluorometry:

Having confirmed the presence of the expected fusion proteins IGF-II-GFP and IGFBP 6-RUC, and the function of the donor luciferase and acceptor fluorophore, cell extracts from these transiently transfected cells were used to carry out a protein binding assay based on energy transfer between the *Renilla* luciferase and 'humanized' GFP moieties of the fusion proteins. Forty-eight hours after calcium transfection, the COS cells were washed twice with PBS and harvested using a cell scraper in luciferase assay buffer containing 0.5 M NaCl, 1 mM EDTA and 0.1 M potassium phosphate at a pH 7.5. The harvested cells were sonicated 3 times for 10 seconds with an interval of 10 seconds using a Fisher Model 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, US) to produce cell extracts.

Next, the cell extracts containing IGF-II-GFP and IGFBP 6-RUC were mixed and 0.1 μg of coelenterazine was immediately added. Spectrofluorometry was performed using a SPEX FluoroMax® (Instruments S.A., Inc., Edison, NJ). The spectrum showed a single emission peak at 471 nm, which corresponds to the known emission of *Renilla* luciferase.

Following the first spectrofluorometry, the mixtures were kept at room temperature for 30 minutes and the spectra were traced again after fresh coelenterazine was added. The trace at 30 minutes showed two peaks with emission maximum at 471 nm and 503 nm. The spectrofluorometry of the cell extracts was carried out at a longer time, but the

spectral pattern did not change over time.

Control cell extract mixtures from cells transfected with pC-INS-GFP and pC-IGFBP 6-RUC were made similarly and their spectra traced. The traces showed only one peak at 471 nm, which corresponds to the emission peak of *Renilla* luciferase. The spectral pattern did not change over time.

Therefore, these data demonstrated that IGFBP 6 and IGF-II interacted but that insulin and IGFBP 6 did not interact.

In addition to the above disclosed examples, protein-protein interactions were also detected by the detection of LRET using corresponding methods in *E. coli* cells and mammalian cells which were co-transformed.

Although the present invention has been discussed in considerable detail with reference to certain preferred embodiments, other embodiments are possible. For example, the interaction between molecules other than proteins could be studied by corresponding methods. Such other molecules could be provided to the living cell by diffusion, infusion, and incorporation or by other means. Further, fusion proteins produced from genetically engineered living cells could have post translational changes, such as the addition of sugar moieties, before their interactions are studied. Also, living cells can be visualized using these methods by spectrofluorometry by low light image analysis in cells, colonies and tissues. Additionally, high through put screening of colonies can be accomplished using the present methods combined with cell sorting and low light video analysis of micro titre dishes or multiple array detection. Therefore, the spirit and scope of the appended claims should not be limited to the description of preferred embodiments contained herein.